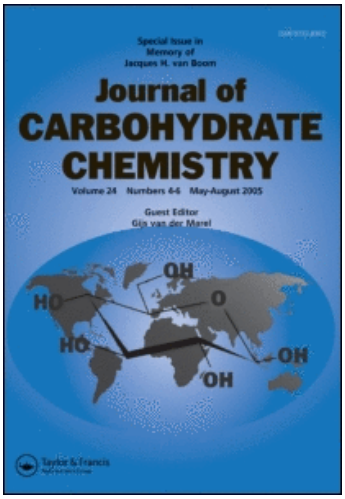

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A HIGHLY SENSITIVE HPLC METHOD TO DISCRIMINATE ENANTIOMERIC AMINO DEOXY SUGARS BASED ON THE DERIVATIZATION WITH (*S*)-(+)-TBMB CARBOXYLIC ACID

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ABSTRACT

A picomole determination of the absolute configurations of amino deoxy sugars was demonstrated through the derivatization at the anomeric carbon with a fluorescent chiral reagent, (*S*)-(+)-TBMB carboxylic acid, followed by the HPLC separations of the derived diastereomeric isomers. A new chemical procedure was developed to convert amino deoxy sugars into per-*NH,O*-acetylated glycosyl chlorides prior to derivatization with the reagent.

INTRODUCTION

A variety of amino deoxy sugars has been identified as components of antibiotics such as streptomycin, kanamycin, hikijimycin, and so on. Identification of their absolute configurations is necessary in determining the correct

structure of the parent antibiotics and is also valuable in studying the antibiotic biosynthetic pathways. Previously,[1,2] we reported a new approach to identify the absolute configurations of neutral monosaccharides based on their anomeric carbon derivatization with a fluorescent chiral derivatizing reagent, (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [(*S*)-TBMB carboxylic acid].[3-6] The method involved a chemical pathway to convert reducing sugars into fluorescent 1-(*S*)-TBMB carboxylates followed by ^{1}H NMR or HPLC analysis. This method, however, could not be applied to 2-acetamido-2-deoxy sugars because the derivatization did not proceed. In the present study, we have reinvestigated the derivatization procedure in order to establish a common method for neutral sugars and for amino deoxy sugars. Here, we wish to report such an improved method along with the HPLC behaviors of a variety of enantiomeric amino deoxy sugars as their 1-(*S*)-TBMB carboxylates. The application is also demonstrated with kanamycin bearing 3-amino- and 6-amino-6-deoxy-D-glucose as the sugar components.

RESULTS AND DISCUSSION

Our preceding method[1,2] involved a chemical conversion of reducing sugars into per-*O*-acetylated glycosyl bromides which were coupled with (*S*)-(+)- or (*R*)-(-)-TBMB carboxylic acid in a basic media. The extension of this chemical method to 2-acetamido-2-deoxy-D-glucose (GlcNAc) and -D-galactose (GalNAc) gave no fluorescent derivative, probably because of the decomposition of the bromides into oxazolines[7] or 1-acetates.[8] Our preliminary test using 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[8] indicated that the 1-chloride could tolerate the reaction conditions to give the β-1-(*S*)-TBMB carboxylate in S$_N$2 process (*ca.* 80% yield). This

result encouraged us to use 1-chlorination instead of 1-bromination for derivatization of amino deoxy sugars.

The reported procedures for the preparations of per-*O*-acetylated glycosyl chloride, however, did not satisfy our analytical purpose; one method[8] utilized gaseous HCl in refluxing ether for acetylated sugars, while another method[9] using acetyl chloride (AcCl) for free sugars took too long reaction time (four days in our experiment). Here, we modified the latter reaction since the reported procedure was simple in handling and seemed to be suitable for the analytical purpose. After having investigated the reaction conditions, we found that the addition of AcOH to the AcCl reaction accelerated the acetylation substantially. The optimized ratio of AcCl:AcOH (5:3) was found after changing the ratio from 10:1 to 1:10. Acetylation could be completed within 1.5 h at 50 ℃ along with partial 1-chlorination. Addition of AcCl and aqueous HCl after the acetylation resulted in accelerating the 1-chlorination due to the increase in the HCl concentration.

As the consequence, we could establish a chemical procedure to convert both neutral and amido deoxy sugars into the per-*NH,O*-acetylated glycosyl chlorides. The typical procedure was as follows **(Figure 1)**.

1) To a mixture of neutral and amino deoxy sugars (maximum amount up to 20 mg) in a test tube was added methanol (1 mL) containing triethylamine (100 μL) and acetic anhydride (50 μL). The mixture was stirred for 0.5 h at room temperature.

2) After the evaporation of the solvents, the residue was reacted with acetic acid (AcOH, 500 μL) and AcCl (300 μL) at 50 ℃ for 1.5 h with occasional stirring.

3) After the solution was cooled to 0 ℃, AcCl (700 μL) and cooled aqueous 12N HCl (150 μL) were added successively, and the mixture was kept at room temperature overnight (8-15 h).

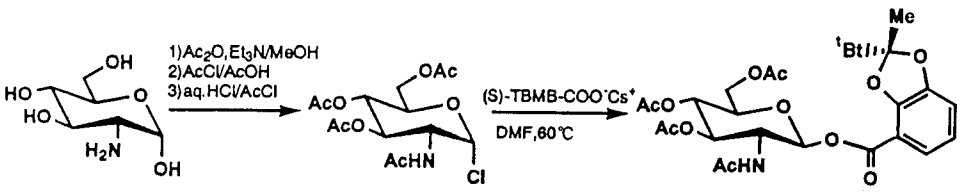

Figure 1. Derivatization of amino deoxy sugar with (*S*)-TBMB carboxylic acid.

4) After evaporation of the solvent, the residue was dissolved in $CHCl_3$ (3 mL). The organic layer was washed with water (2 mL x 3) and aqueous $NaHCO_3$ (2 mL x 3), and dried over $MgSO_4$. This solution containing per-*NH*,*O*-acetylated glycosyl chlorides could be used for the coupling with (*S*)- or racemic TBMB carboxylic acid in the next steps [(5) and (6)] or stored for several weeks in a refrigerator below 4 ℃.

The procedures (1) and (2) may be replaced by one step per-*NH*, *O*-acetylation in acetic anhydride in pyridine. In this case, however, removal of pyridine should be performed before the 1-chlorination in the next step (3). Thus, the two step procedures in the present study can be completed in a more facile manner. The TLC analysis for the final $CHCl_3$ solution in the procedure (4) indicated that more than 90% of per-*NH*,*O*-acetylated sugars were converted into 1-chlorides for all sugars examined here. Allowing the solution to stand for a prolonged time at the third step did not effectively increase the amount of 1-chlorides, probably because there existed an equilibrium between 1-chlorides and the 1-acetates in the obtained HCl-AcOH solution. In the above procedure, moisture in a sample or in a test tube did not affect the reaction. On the contrary, a small content of water was found to slightly accelerate the 1-chlorination at the second step, possibly

because water was consumed by AcCl or acetic anhydride to give a HCl-AcOH solution.

The coupling of 1-chlorides with (*S*)-TBMB carboxylic acid was performed in a similar manner to our previous method (**Figure 1**). Here, cesium (*S*)-TBMB carboxylate was employed in dimethylformamide (DMF) in order to simplify the procedure and activate the reaction instead of using (*S*)-TBMB carboxylic acid and potassium hydrogencarbonate in acetone. The typical procedure is summarized as follows.

5) An aliquot of the $CHCl_3$ solution from procedure (4) was placed in another test tube, and the solvent was evaporated. To the residue was added 0.1 M cesium (S)-TBMB carboxylate in DMF (300 μL, more than 10 fold excess over the expected sugar amount). The mixture was heated at 60 ℃ for 3 h with occasional stirring.

6) An aliquot (10 μL) of the reaction solution was diluted with CH_3CN (200 μL), and 0.2 μL of the diluted solution was subjected to HPLC analysis (ODS column, 150 x 4.6 mm i.d., mobile phase: CH_3CN:H_2O:*iso*-PrOH = 3:7:2, flow rate = 0.5 mL/min, fluorescent detection at Ex = 310 nm and Em = 380 nm).

By using the above procedure, a 1:1 mixture of galactose and galactosamine-HCl salt gave a 1:1 mixture of corresponding 1-(*S*)-TBMB carboxylates in [**Figure 2(C)**], showing a clear contrast to the HPLC chart [**Figure 2(A)**] by our previous method which gives no HPLC peak corresponding to galactosamine. Our previous ^1H NMR and HPLC studies[1,2] were applied to determine the stereochemistries of the products. These showed that the present reaction condition gave selectively 1,2-*trans* TBMB carboxylates from sugars with an *equatorial* C-2 substituent (OAc, NHAc) like GlcNAc, GalNAc, Glc and Gal, while it gave a mixture of 1,2-*trans* and *cis* isomers from sugars with an *axial* C-2 group such as Man and

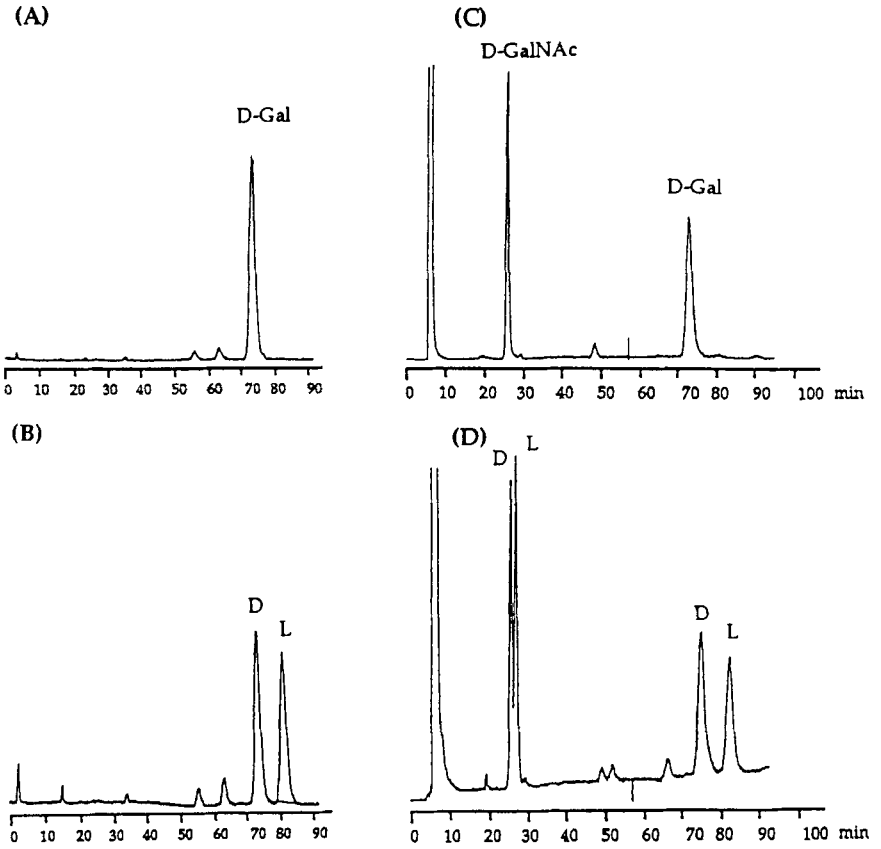

Figure 2. HPLC Analysis of a mixture of galactose and 2- acetamido-2-deoxy-galactose.
(A) The analysis of the 1:1 mixture based on the HBr-AcOH method (*lit.* 1,2) using (*S*)-TBMB carboxylic acid, (B) using racemic TBMB carboxylic acid, (C) processed based on the present AcCl-AcOH method using (*S*)-TBMB carboxylic acid and (D) using racemic TBMB carboxylic acid. (D,L-Assignments were based on the assumption that D,L-sugars were used for the analysis. HPLC conditions were cited in the text).

ManNAc. The reactions on a preparative scale showed that the derivatization of the former sugars proceeded in higher yields (70-90%) than that of the latter (10-30%). Thus, the stereochemical outcomes were similar to those observed in the 1-bromination method except for glucose and xylose which gave

consistently an unidentified small (5%~20%) HPLC peak in front of the main HPLC peak of the 1,2-*trans* isomer. This small peak may be due to the 1,2-*cis* isomer or the furanose isomer.

When a racemic reagent was used for the derivatization of D-sugars, a 1:1 mixture of diastereoisomeric 1-(*S*)- and 1-(*R*)-TBMB carboxylated sugars was obtained. For the sugars studied here, the two diastereomers could be well separated from each other under reversed-phase HPLC conditions. This means that D,L-enantiomers of these sugars can be separated in the same way by using an optically pure (*S*)- or (*R*)-reagent. The HPLC data in the **Table** were summarized assuming the use of D,L-sugars and (*S*)-TBMB carboxylic acid for the analytical purpose. The data clearly showed that the elution order of D- and L-enantiomers was governed by the anomeric configurations of the 1-(*S*)-TBMB carboxylated derivatives; the (*1S*)-isomer was always eluted faster than the (*1R*)-isomer. This rule which is valid for any neutral sugars[2] can be used to assign the absolute configuration of sugars since the derivatization reaction proceeded stereoselectively to give 1,2-*trans* isomers with the exception of sugars with an *axial* C-2 substituent. For such exceptional sugars, the second peaks of the 1,2-*trans* isomers will be diagnostic for identification of their D,L-configuration based on the above rule.

The detection limit of the 1-(*S*)-TBMB carboxylated sugars was *ca.* 0.2 picomole as the HPLC injection amount (S/N = 3). Thus, a highly sensitive assignment of the absolute configurations has become possible for any neutral and amino deoxy sugars studied here and previously[2] in this way.

APPLICATION to KANAMYCIN

The method described above was applied to the analysis of kanamycin. Kanamycin has two amino deoxy sugars, 6-amino-6-

Table. HPLC data of 1-*O*-(*S*)-TBMB carboxylated D,L-amino deoxy sugars.

	Amino sugars		Rt (min)	Configurations	α^a	Rs^b
1	$Glc2NH_2$		19.68	1S (D)	1.05	1.00
			20.82	1R (L)		
2	$Glc3NH_2$		27.51	1S (D)	1.07	1.50
			29.55	1R (L)		
3	$Glc4NH_2$		30.25	1S (D)	1.08	1.74
			32.72	1R (L)		
4	$Glc6NH_2$		32.53	1S (D)	1.10	2.20
			35.89	1R (L)		
5	$Man2NH_2$	1,2-*trans*	25.27	1S (L)	1.07	1.18
			27.14	1R (D)		
		1,2-*cis*	18.97	1S (D)	1.07	1.06
			20.24	1R (L)		
6	$Gal2NH_2$		20.24	1S (D)	1.06	1.08
			21.42	1R (L)		
7	$Gal3NH_2$		28.61	1S (D)	1.07	1.28
			30.57	1R (L)		
8	$Xyl3NH_2$		23.19	1S (D)	1.07	1.44
			24.84	1R (L)		

HPLC conditions: ODS column (150 mm × 4.6 mm ϕ). Solvents: CH_3CN:H_2O:*iso*-PrOH=3:7:2. Flow rate=0.5mL/min.

a. Separation factor = (retention volume of one diastereoisomer - void volume of the column)/(retention volume of another diastereoisomer - void volume of the column).

b. Resolution factor = 2× (distance between the peaks of two diastereoisomers)/(sum of the band width of the two peaks).

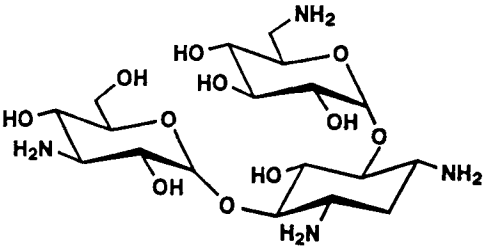

Figure 3. Structure of kanamycin.

deoxy and 3-amino-3-deoxy-D-glucose and a 2-deoxy streptamine (**Figure 3**). Although the absolute structure has long been established by both chemical degradations[10] and the X-ray analysis,[11] we have used kanamycin to demonstrate the simplicity of our present method for determination of the absolute configurations of sugar components in antibiotics.

Kanamycin disulfate was hydrolyzed in a sealed test tube (2N aq HCl, 100 ℃) to afford a mixture of amino deoxy sugars (for details see Experimental). After the evaporation of the solvents, the residue was subjected to the chemical proceduces described in the preceding section for the 1-(*S*)- or racemic TBMB derivatizations. The HPLC charts of the kanamycin hydrolyzates derivatized with (*S*)-TBMB reagent **[Figure 4(C)]** gave two main peaks corresponding to 3-amino and 6-amino-6-deoxyglucose. By the comparison with the HPLC chart **[Figure 4(D)]** derived from racemic TBMB reagent, it was revealed that these two peaks matched with those of the faster eluted diastereomers with a (*1S*)-configuration at the anomeric center. By applying the empirical HPLC rule described in the preceding section, the absolute configuration of the two amino deoxy sugars could be assigned to be D-form. This result could be further confirmed by using authentic 3-amino-3-deoxy- and 6-amino-6-deoxy-D-glucose prepared from D-glucose according to reported methods.[12-17]

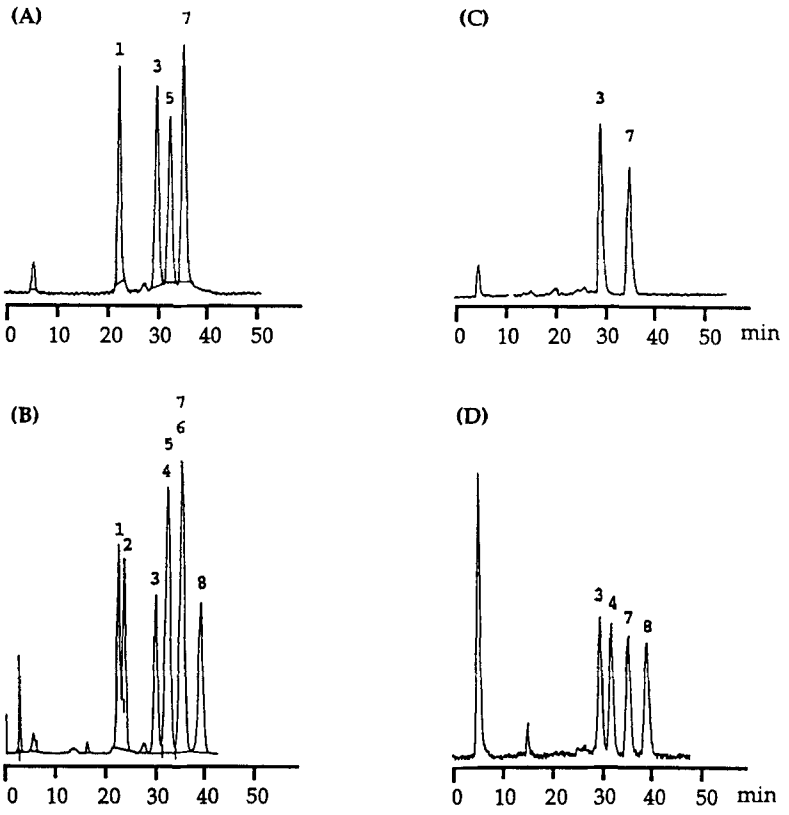

Figure 4. HPLC Profiles of a variety of amino deoxy glucoses processed based on the present AcCl-AcOH method.
Charts (A) and (B); a standard mixture of 1-*O*-[(*S*)-TBMB carbonyl] derivatives. Charts (C) and (D); amino deoxy sugar components derived from kanamycin based on the procedure in the text. Peak assignments; 1=2-amino-2-deoxy-D-glucose, 2=2-amino-2-deoxy-L-glucose, 3=3-amino-3-deoxy-D-glucose, 4=3-amino-3-deoxy-L-glucose, 5=4-amino-4-deoxy-D-glucose, 6=4-amino-4-deoxy-L-glucose, 7=6-amino-6-deoxy-D-glucose, 8=6-amino-6-deoxy-L-glucose.

On the HPLC chart in **Figure 4(C)**, the amino cyclitol component (2-deoxystreptamine) of kanamycin did not appear. This result indicated that the TBMB-derivatization employed here occurred selectively for reducing sugars by a nucleophilic substitution reaction at the reactive anomeric

carbon. **Figure 4(A and B)** showed that each amino deoxy sugar with an amino group individually at C-2, C-3, C-4 and C-6 was clearly separated under the present HPLC conditions. Moreover, amino deoxy sugars were eluted faster than neutral sugars. These results suggest that the present HPLC approach based on the derivatization with (*S*)-TBMB carboxylic acid may be applied to more complicated sugar mixtures derived from oligo- and polysaccharides by optimizing the HPLC conditions.

CONCLUSION

We have developed a general HPLC method to determine the absolute configuration of both neutral and amino deoxy sugars. A previous approach *via* the 1-bromination of sugars[1,2] is still valid especially for the analysis of neutral sugars or even for amino deoxy sugars without a C2-NH_2 group since the reaction can be completed more quickly and generally in higher yields compared with the present approach *via* the 1-chlorination. The use of the present method is particularly valuable when 2-amino-2-deoxy sugars are expected to exist in the samples and can be generally recommended since the procedure can be more easily performed compared with the previous method using a more difficult to handle HBr solution in acetic acid.

EXPERIMENTAL

Equipment. ^1H NMR spectra were measured using a JEOL GX 400 spectrometer at room temperature in D_2O or $CDCl_3$ solution with 3-(trimethylsilyl)propionic acid-d_4 sodium salt or tetramethylsilane as an internal standard. High resolution mass (HRMS) spectra (EI-MS, 70eV) were recorded with a JEOL JMS-DX303HF in lieu of elemental analysis. The

$[\alpha]_D^{20}$ was measured on a JASCO DIP-4 digital polarimeter. HPLC separations were conducted with a CCPM multipump (Tosoh, Tokyo, Japan) connected to a Tosoh model (Fs-8000 fluorescent detector monitoring at λex= 310 nm and λem= 380 nm). Separations were performed on a C-18 column (Capcell pak, 4.6mmϕx150mm, Shiseido, Tokyo, Japan). Analyses were carried out isocratically using a mixture of CH_3CN, H_2O and *iso*-PrOH (3:7:2, v/v; 0.5mL/min) as the mobile phase at room temperature, and peaks were recorded with a model 807-IT integrator (JASCO).

Chemicals. For the HPLC analysis, H_2O of HPLC grade (Kanto Chemical CO., INC. Tokyo, Japan) and super-special pure grade *iso*-PrOH (Wako Pure Chemical Industries, LTD., Osaka, Japan) were used as received. CH_3CN was used after distillation. Kanamycin and common sugars were purchased from Merck and Wako Pure Chemical Industries, LTD., (Osaka, Japan) respectively. Racemic and (*S*)-TBMB-COOH were synthesized according to our method described previously.[3-5] 3-Amino-3-deoxy-D-glucose, 4-amino-4-deoxy-D-glucose, 6-amino-6-deoxy-D-glucose and 3-amino-3-deoxy-D-xylose were synthesized according to reported methods.[12-16]

Cesium (*S*)-TBMB carboxylate. (*S*)-TBMB-COOH (500 mg, 2.1 mmol) and Cs_2CO_3 (344.2 mg, 1.05 mmol) were added to 5 mL of MeOH. This mixture was stirred for 3 h at room temperature. After removing the solvent by evaporation *in vacuo*, (*S*)-TBMB-COO^-Cs^+ was obtained as solid; $[\alpha]_D^{20}$ +23.3° (*c* 0.94, MeOH); ^1H NMR (D_2O), 1.08 (s, 9H), 1.62 (s, 3H), 6.84 (dd, J=7.6 and 7.6Hz, 1H), 6.89 (dd, J=7.6 and 2.4Hz, 1H), 7.18 (dd, J=7.6 and 2.4Hz, 1H). HRMS Calcd for $C_{12}H_{12}O_4Cs$ (352.9785). Found: 352.9813. mp $\geqq$ 280 ℃ (decomposed).

Racemic cesium TBMB carboxylate. The racemic salt was prepared in the same manner as described above by using racemic TBMB-COOH and Cs_2CO_3 in MeOH.

Acid hydrolysis and derivatization of kanamycin. Kanamycin disulfate (5 mg) was heated in a sealed test tube with 6N aqueous HCl (1 mL) at 100 ℃ for 10 h. After the solution was cooled at 0 ℃, the tube was opened and the solution was concentrated *in vacuo*. To a solution of the residue in MeOH (1 mL), Et_3N (40 μL) and acetic anhydride (20 μL) were added, and the mixture was stirred at room temperature for 0.5 h. After solution concentration, the residue was treated with a mixture of acetic acid (0.5 mL) and AcCl (0.3 mL) at 50 ℃ for 1.5 h. AcCl (0.7 mL) and cooled aqueous 12N HCl solution (0.15 mL) were added at 0 ℃, and the mixture was kept at room temperature overnight. After evaporation of solvent, the residue was dissolved in $CHCl_3$ (3 mL), washed with water and aqueous $NaHCO_3$ and then dried over $MgSO_4$.

A small portion (100 μL) of the $CHCl_3$ solution separated in another test tube was concentrated to dryness and mixed with cesium (*S*)-TBMB carboxylate in DMF solution (0.1 M, 300 μL). The mixture was heated at 60 ℃ for 3h. A small aliquot (10 μL) of the mixture was diluted with MeOH (100 μL), from which 0.2 μL were injected into the HPLC column.

The same procedure from the $CHCl_3$ solution (100 μL) as described above was performed by using racemic cesium TBMB carboxylate.

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